Coffee and chemoprevention – the genotype decides

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Abstract

In a human intervention study comprising 49 healthy participants, coffee combining natural green coffee bean constituents and dark roast products was identified as a genotype-dependent inducer of Nrf2, significantly affecting Nrf2 gene expression and downstream transcription. Specifically, with 65% of participants showing ≥1.5 fold increase in Nrf2-transcription, the presence of the -651G/A SNP in the Nrf2 gene in conjunction with heterozygosity of the 6/7 AT repeat sequence in the UGT1A1 gene significantly down-regulated coffee-mediated gene expression. Considering the role of the Nrf/ARE pathway in the regulation of antioxidative and chemopreventive phase II efficacy, individual genotype must be considered when examining the potency of bioactive food/food constituents and therapeutic potential.

Keywords:

Reactive oxygen species, antioxidant, coffee, Nrf2 genotype, genetic variations, gene expression

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Introduction

Phase II enzymes play a critical role in converting reactive electrophiles or xenobiotics into less toxic products and seem to be crucial for cancer prevention, with deficiency in phase II enzymatic activity associated with increased risk of colon cancer [1, 2]. The expression of many phase II genes is regulated via the activation of antioxidant response elements (ARE), located in the 5'-flanking region of their respective promoters. Activation by ROS or upstream protein kinases induces translocation of the transcription factor Nrf2 (Nuclear factor-erythroid 2 p45 subunit (NF-E2)-related factor 2) from the cytoplasm into the nucleus, binding to ARE and subsequent transcription of phase II enzymes, including glutathione *S*-transferases (GST), UDP-glucuronyl transferases (UGT) or γ-glutamyl cysteine ligase (γGCL) [3-12]. During a human intervention trial, we recently, identified the typical dark roast coffee constituent *N*-methylpyridinium (NMP) as well as the green bean constituent n-chlorogenic acid (CGA), as potent activators of the Nrf2/ARE pathway [13]. Over four weeks, participants consumed the study coffee, a blend combining both these constituents (typical dark roast characteristics as NMP with considerable amounts of green bean constituents) resulting in a significant increase in mean Nrf2 gene transcripts in peripheral blood lymphocytes (PBLs) [14, 15]. However, large differences in Nrf2 activation ability were identified in participants, which could not be explained by differences in their general health status or nutritional behavior.

Previous reports have highlighted genetic variation in specific antioxidative genes determine their ability to be activated by bioactive food compounds [16]. In addition, biochemical studies have associated allelic variations in the Nrf2 promoter region with differences in antioxidative phase II gene transcription [17]. To date, 9 single nucleotide polymorphisms (SNPs) have been identified in the Nrf2 gene [17-19]. Of relevance are the -617C/A and the -651G/A SNPs, located within the promoter region. Both SNPs have been shown to reduce Nrf2 transcriptional activity reflected by attenuated binding of Nrf2 to the ARE, resulting in decreased Nrf2 transcription [17]. Genotyping of a subset of individuals from our previous study indicated a connection between Nrf2 genotype and response to coffee [15]. Based on these findings, we undertook the current intervention trial to (a) test Nrf2-activation potential of the study coffee in a larger cohort; and (b) to determine whether individual differences in response to the study coffee is associated with specific genotypes of

the Nrf2, GST1A1 and UGT1A1 genes.

Results

Nrf2 genotype and gene transcription

A 424bp polymorphic region of the Nrf2 promotor was sequenced in all 49 participants of the intervention trial to examine the -617C/A, the -653A/G and the -651G/A polymorphisms, already determined to be functionally relevant for transcriptional activity of the Nrf2 gene [17]. The -617C/A SNP was present in 10/49 (20%) of participants (Table 1). The presence of the -651G/A SNP was observed in 15/49 individuals (31%) while 31/49 (63%) of the participants possessed a SNP at position -653A/G.

Table 1

Genotype distribution of Nrf2, GST1A1 and UGT1A1 in participants of the coffee intervention study

Gene	SNP	Frequency		Literature		Ref.
		n	[%]	N	[%]	
Nrf2	**-617C/C (WT)**	39/49	80	16/20	80	[17]
	-617C/A	10/49	20	4/20	20	[17]
	-651G/G (WT)	34/49	69	18/20	90	[17]
	-651G/A	15/49	31	2/20	10	[17]
	-653A/A (WT)	18/49	37	15/20	75	[17]
	-653A/G	31/49	63	5/20	25	[17]
GST1A1	**A/A (WT)**	24/48	50	106/27	38	[20]
	A/B	18/48	38	133/27	48	[20]
	B/B	6/48	12	39/278	14	[20]
UGT1A1	**$[TA]_6TAA$ (WT)**	21/46	46	183/399	46	[23]
	$[TA]_{6/7}TAA$	19/46	41	169/399	42	[23]
	$[TA]_7TAA$	6/46	13	47/399	12	[23]

Changes in Nrf2 gene transcription were also assessed at the different blood collection points (CPs) in the course of the study. A potent induction of Nrf2 gene transcription after four weeks consumption of the study coffee (BC3) in comparison to

both wash-out (BC2) as well as normal diet (BC1) was demonstrated (Figure1A). In total, 65% (32/49) of all participants displayed a ≥1.5-fold induction of Nrf2 gene transcription following coffee consumption. When we more closely examined the genotype-dependent magnitude of the Nrf2 activation, it was clear that the -651 SNP carriers displayed a weaker Nrf2 activation after coffee consumption (BC3) when compared to WT and the other genotype combinations (Figure 1B). Comparing the sensitivity of the response between all genotypes demonstrated that individuals carrying both the -617 and the -651 SNP concomitantly possessed the lowest potential to activate Nrf2 gene transcription (see supporting information, Figure S1).

Figure 1A

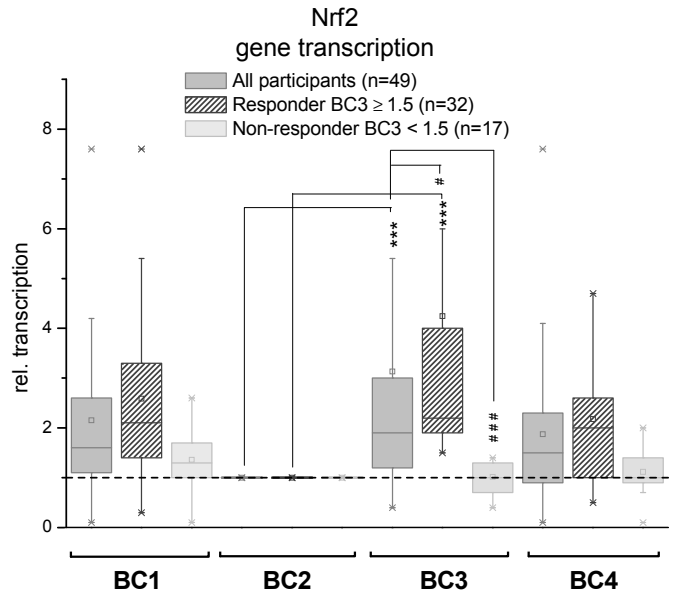
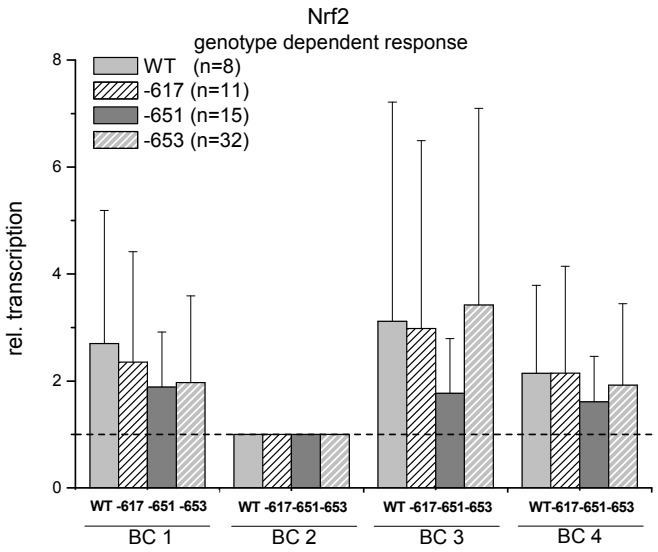


Figure 1B



Legend Figure 1

(A) Modulation of Nrf2 gene transcription in PBLs of 49 participants of the intervention trial (samples of two participants failed to show sufficient fluorescence signals/amplification). The data, analyzed in triplicate are presented as BOX-diagrams, normalized to ß-actin expression and as relative transcription of individual levels of each participant before the study (BC1), after a four week wash-out (polyphenol-poor diet and no coffee consumption, BC2), after four weeks of daily 750 mL coffee consumption (BC3), after a second wash-out period of four weeks (BC4). The data are represented as relative transcription of BC2=1. BC: blood collection. (B) Nr2 genotype dependent magnitude of changes in Nrf2 gene transcription during the course of the trial. The data are the mean ± SD of the individuals, grouped according to their respective genotypes and presented as relative transcription of BC2 (wash-out) =1. Significances indicated are calculated by Student's *t* test ($^{\#}p<0.05$ and ***, $^{\#\#\#}p<0.001$). BC: blood collection.

GST1A1 genotype and gene transcription

We next examined the GST1A1 polymorphism comprised of the two alleles, GST1A1*A and GST1A1*B [20]. The two genotypes differ in three linked base changes in the 5'-regulatory region of the gene (a T/G change at -567, a C/T change at -69 and G/A change at -52). Presence of the C/T base change at position -69 (GST1A1*B) gives rise to an *Ear1* restriction site, resulting in two fragments of 380bp and 100bp following digestion. GST1A1*A homozygotes show only one band at

480bp (see supporting information, Figure S2). From the intervention trial, 24/48 (50%) of participants were found to carry the A/A genotype, 18/48 (38%) the heterozygous A/B genotype and 6/48 (12%) the rare homozygous B/B genotype (Table 1). We then investigated whether four weeks of intervention with the study coffee modulated GST1A1 transcription levels, an important member of the antioxidative GST enzyme family. GST1A1 gene transcription was potently elevated in the majority of participants after four weeks consumption of coffee (BC3) in comparison to the wash-out phase (BC2, Figure 2A). Stratification of participants with respect to coffee response demonstrated that 54% of study participants displayed a ≥1.5-fold induction of GST1A1 transcription following coffee consumption. Furthermore, this group termed “coffee responders”, also displayed a higher induction of Nrf2 transcription during the BC1 phase of the study where participants followed their normal diet (Figure 2A). When we compared GST1A1 genotype in relation to changes in GST1A1 transcription, no significant difference in GST1A1 activation was evident. However, individuals possessing the homozygous BB genotype appeared to be slightly more susceptible to changes in GST1A1 gene expression following coffee consumption (see supporting information, Figure S3). The GST1A1 gene carries an ARE sequence in its promotor region, hence its transcription is activated by Nrf2. Our data clearly demonstrates the transcriptional activity of Nrf2 and subsequently the ability to activate ARE-dependent genes appears to be dependent on the specific Nrf2 genotype of the individual. In addition, there appears to be a magnitude effect of Nrf2 genotype-dependent GST1A1 activation. Participants carrying the -651SNP (31% of the participants), showed no increase in GST1A1 gene transcription after coffee consumption (Figure 2B). Thus, individuals, possessing the -651 SNP in their Nrf2 gene were disadvantaged in the antioxidative potential of the study coffee in comparison to individuals lacking this SNP, indicating the importance of Nrf2 genotype on antioxidative GST1A1 response.

Figure 2A

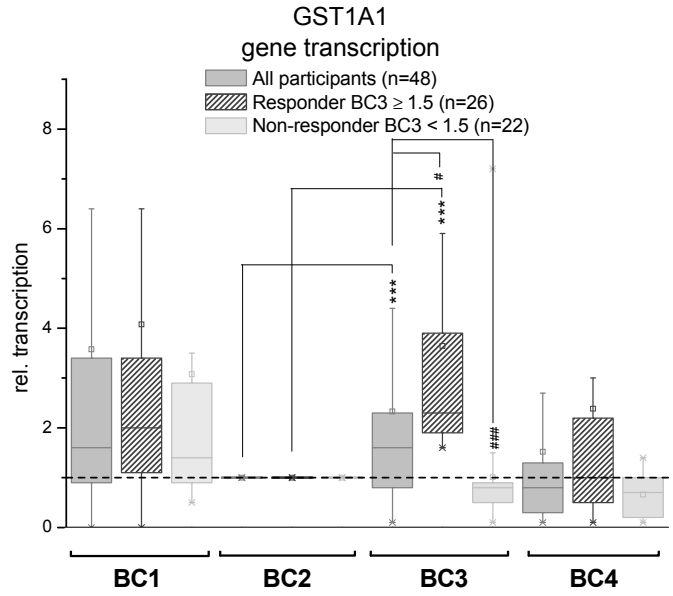
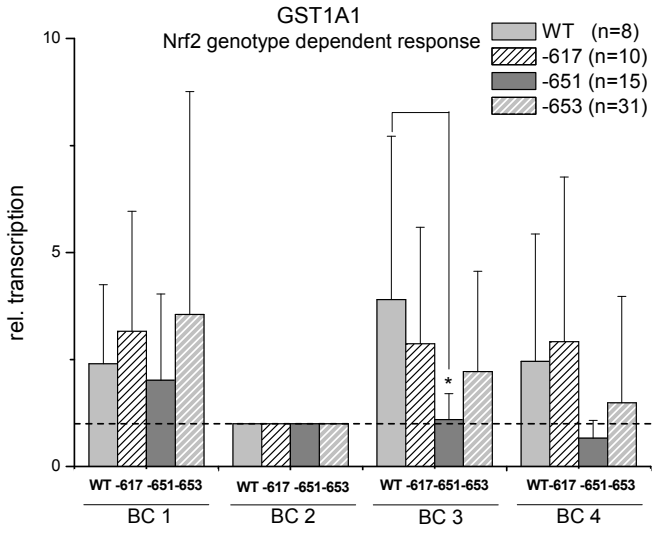


Figure 2B



Legend Figure 2

(A) Modulation of the GST1A1 gene transcription in PBLs of 48 participants of the intervention trial (samples of three participants failed to show sufficient fluorescence signals/ amplification). The data, performed in triplicate are presented as BOX-diagrams, normalized to ß-actin expression and as relative transcription of individual levels of each participant before the study (BC1), after a four-week wash-out (polyphenol-poor diet and no coffee consumption, BC2), after four weeks of daily 750mL coffee consumption (BC3), after a second wash-out period of four weeks

(BC4). The data are represented as relative transcription of BC2=1. BC: blood collection. (B) Nrf2 genotype dependent magnitude of changes in GST1A1 gene transcription during the course of the human intervention trial. The data are the mean ± SD of the individuals, grouped according to their respective genotypes and presented as relative transcription of BC2 (wash-out) =1. Significances indicated are calculated by Student's *t* test (*, $^{\#}p<0.05$ and ***, $^{\#\#\#}p<0.001$). BC: blood collection.

UGT1A1 represents another important phase II gene in cellular detoxification processes. With activation of UGT1A1 gene transcription previously reported in a murine model [21], we next examined changes of UGT1A1 gene transcription in the study participants. UGT1A1 possesses a known variation in its promotor sequence [22], with an additional TA repeat in the TATA region (7 TA repeats; UGT1A1*28) known to markedly decrease UGT1A1 expression resulting in reduced activity [22, 23]. Most participants of the present intervention trial were found to possess either a homozygote $[TA]_{6/6}$-repeat sequence (wild type, 21/46 individuals (46%)) or were heterozygous ($[TA]_{6/7}$ (19/46 individuals (41%)), whereas only 13% (6/46) of the individuals were identified as homozygous $[TA]_{7/7}$-repeat sequence carriers (Table 1). Although a wide variation in UGT1A1 transcription levels amongst participants was detected at the early timepoint (BC1, Figure 3A), the affect on transcription in response to coffee following adherence to the trial conditions was more evident at the BC3 stage of the trial. On average, induction of UGT1A1 gene transcription was observed in participants after four weeks intervention with the study coffee. Furthermore, the absence of coffee during the second wash-out period (BC4) resulted in down-regulation of UGT1A1 gene expression to basal levels, emphasizing the study coffee constituents crucial for the expression induced at BC3. When we stratified the participants into responders (BC3 ≥1.5) and non-responders (BC3 <1.5), 63 % of individuals were identified as coffee responders, supporting our previous findings on activation of UGT1A1 gene transcription by the study coffee. Although UGT1A1 transcription was previously defined in a murine model, this is the first time this has been demonstrated in humans (Figure 3A). We then correlated changes in UGT1A1 transcription with participant UGT1A1 genotype during the response to coffee intervention period (BC3). This revealed a clear and significant correlation between reduced response to coffee in the presence of the 6/7 heterozygous

genotype (Figure 3B). Individuals carrying either the homozygous 6/6 or the heterozygous 7/7 genotype appeared to respond equally to the study coffee. Correlating UGT1A1 gene transcription with Nrf2 genotypes indicated the -651 Nrf2 genotype was associated with a lower coffee-mediated increase in gene transcription. In addition, these individuals showed no increase in UGT1A1 gene expression following coffee consumption at BC3 when compared to the wash-out at BC2. In contrast, WT individuals or -617 or -653 SNP variants displayed a marked increase of UGT1A1 gene transcription following coffee consumption (Figure 3C). Linking participant response to the study coffee and genotype, clearly demonstrated that 63% (5/8) of individuals carrying both the 6/7 UGT1A1 and the -651 Nrf2 genotype belonged to the group of coffee non-responders, suggesting a strong correlation between genetic background and response to coffee.

Figure 3A

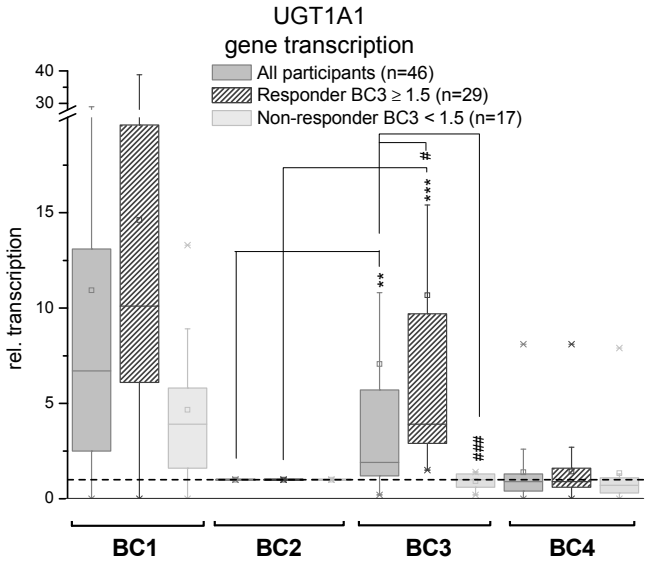


Figure 3B

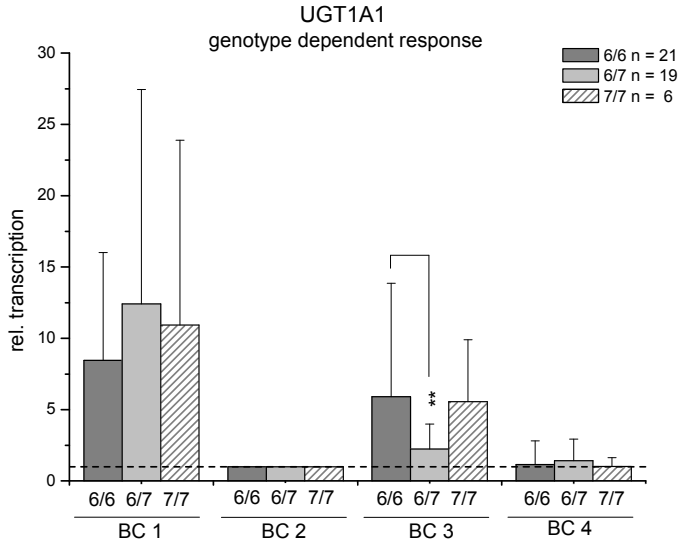
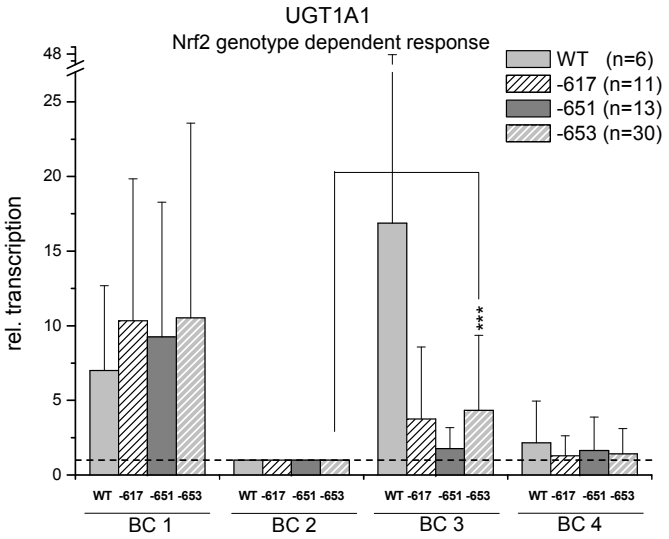


Figure 3C



Legend Figure 3

(A) Modulation of the UGT1A1 gene transcription in PBLs of 46 participants of the human intervention trial (samples of five participants failed to show sufficient fluorescence signals/ amplification). The data, performed in triplicate are presented as BOX-diagrams, normalized to ß-actin expression and as relative transcription of individual levels of each participant before the study (BC1), after a four week wash-out (polyphenol-poor diet and no coffee consumption, BC2), after four weeks of daily 750mL coffee consumption (BC3), after a second wash-out period of four weeks (BC4). The data are represented as relative transcription of BC2=1. BC: blood

collection. (B) UGT1A1 genotype dependent magnitude of changes in UGT1A1 gene transcription during the course of the human intervention trial. The data are the mean ± SD of the individuals, grouped according to their respective genotypes and presented as relative transcription of BC2 (wash-out) =1. (C) Nrf2 genotype dependent magnitude of changes in UGT1A1 gene transcription during the course of the human intervention trial. The data are the mean ± SD of the individuals, c grouped according to their respective genotypes and presented as relative transcription of BC2 (wash-out) =1. Significances indicated are calculated by Student's *t* test (*, $^{\#}p<0.05$, $^{**}p<0.01$ and ***, $^{\#\#\#}p<0.001$). BC: blood collection.

Discussion

In this study, for the first time coffee was identified as a potent inducer of the antioxidative and chemopreventive Nrf2/ARE pathway in humans. This was clearly demonstrated by a significant increase in Nrf2, GST1A1 and UGT1A1 gene transcription after four weeks of coffee consumption in the mean of the 51 participants. Although this trend was clear, differences in individual responses to the study coffee (Figure 1A, 2A, 3A) were also observed. Examining this data more closely, the response of each participant for each of the genes examined revealed a clear group of responders (Nrf2 65% (32/49); GST1A1 54% (26/48) and UGT1A1 63% (29/46)) having a ≥1.5 fold increase in gene transcription following coffee intervention (BC3) when compared to the wash-out period (BC2). A smaller group of non-responders (Nrf2 35% (17/49); GST1A1 46% (22/48) and UGT1A1 37% (17/46)) with a <1.5 fold increase observed in gene transcription following coffee consumption was also identified. These clear differences in gene expression in response to the study coffee, could not be explained by differences in dietary uptake of any antioxidants in participant including general diet, specific foods consumed, coffee intake as well as gender, ethnicity or age. In order to elucidate any genotypic involvement, i.e. the presence of specific allele or alleles influencing response to coffee, we examined regions within the Nrf2, GST1A1 and UGT1A1 genes, carrying known polymorphisms previously demonstrated to affect transcriptional activity [15, 17, 20, 22, 23].

Allele frequencies for the most common Nrf2 SNPs examined were in concordance with current literature and available data (Table 1). The -617C/A SNP was present in

10/49 (20%) of participants and the -651G/A SNP was observed in 15/49 individuals (31%), with 31/49 (63%) of the participants displaying a SNP at position -653A/G. Thus, data from the study demonstrate a higher incidence of the -653 SNP in the Nrf2 promotor. This is in line with previously published data [15], but is higher than data published by Marzec et al. (2007). However, when we combine data from the current study (51 individuals) with the two previous studies (n=20 in Marzec et al (2007) and n=18 in [15] et al) incidence of the -653 SNP is 56-63% in Caucasians.

When we next examined a polymorphism in the GST1A1 gene (alleles GST1A1*A and GST1A1*B [21], 24/48 (50 %) of the participants were identified as A/A carriers, 18/48 (38%) possessed the heterozygous A/B genotype and 6/48 (12%) were identified as B/B carriers (Table 1). The frequency of the GST1A1 genotypes was comparable to other studies in Caucasians [20] indicating the study data appears to represent an average population profile.

The UGT1A1 gene possesses the known variation UGT1A1*28, the incidence of which varies amongst ethnicities, highest in African (43%) or European (54%) descended individuals and lowest in those of Asian (16%) descent [23]. The study population displayed a ratio of genotypes within the expected range (Table 1) with 21/46 individuals (46%) WT ($[TA]_{6/6}$-repeat) and 19/46 participants (41%) heterozygous ($[TA]_{6/7}$-repeat). Of those examined, 6/46 individuals (13%) carried a homozygous $[TA]_{7/7}$-repeat sequence, the low frequency of this genotype is supported by previous data in the literature [23].

Analysing the changes in gene transcription of Nrf2, GST1A1 and UGT1A1 during the course of the two-month trial with respect to individual genotypes revealed several associations. In particular, a genotype-dependent response to the study coffee was observed for the -651 SNP of Nrf2. Individuals carrying this genotype showed a considerably lower magnitude of induction in Nrf2 gene transcription during the coffee intervention period (BC3) when compared to participants carrying the WT sequence (a -617 or -653 SNP) (Figure 1B).

Transcription of the UGT1A1 gene was significantly up-regulated in 63% of participants in this study. The UGT1A1 gene also demonstrated the strongest variation in the response to the study coffee in association with genotype. Both the magnitude of the response, as well as the frequency of induced UGT1A1 gene transcription following coffee consumption was significantly decreased in carriers possessing a heterozygous 6/7 sequence in the promotor region. From the study,

18/51 (35%) individuals carried this genetic modification and most of these individuals failed to increase UGT1A1 gene transcription following coffee consumption (Figure 3B).

Previous studies have reported reduced Nrf2 transcriptional activity affecting downstream ARE-dependent gene transcription in individuals carrying genetic variations in the Nrf2 promotor [17, 18]. In the present study, individuals carrying the SNP at -651 in the Nrf2 gene demonstrated a significantly reduced response to coffee, correlating to a significantly lower induction of Nrf2-dependent GST1A1 and UGT1A1 gene transcription (Figure 2B, 3C). These findings are supported by Marzec et al. (2007) who detected a reduced transcriptional activity of Nrf2, reflected by an attenuated binding of Nrf2 to the ARE (EpRE) in the presence of the -651 SNP. Furthermore, individuals carrying this genetic variation seem to suffer more frequently from acute lung injury (ALI), a disease, related to reduced antioxidative cell defence [17]. In addition, the genotype specific response of the UGT1A1 gene is interesting, as previous research in Gilbert's Syndrome has indicated that the $[TA]_{7/7}$-repeat genotype was associated with increased bilirubin and thus reduced enzymatic activity [22]. That the $[TA]_{6/7}$-repeat genotype displayed reduced induction of the gene in response to coffee in our study may reflect some kind of interference between the alleles in mediating gene response, perhaps some kind of asymmetric recruitment of transcription factors that slows response efficiency not present in homozygous individuals. This may also be a reflection of the relatively low numbers of $[TA]_{6/6}$-repeat individuals in the study reducing our ability to accurately determine the response in this group. This kind of variable response is worthy of additional follow-up in larger cohorts and mechanistic models, to determine its validity. The overall findings of this study demonstrate the influence of genotype on general/physiological gene transcription. We have expanded this to examine the effect of Nrf2 genotype on transcriptional activation after food intervention.

Taken together, the study coffee, combining potent Nrf2 inducers from green coffee beans with typical high roasted constituents, has been identified as a potent inducer of antioxidative cell protection in the mean of participants in a two-month coffee intervention trial. Differences in the coffee response within the study population resulted in the identification of a group of responders and non-responders for transcription of Nrf2, GST1A1 and UGT1A1 in peripheral blood lymphocytes. In

addition, the frequency of the -651 SNP in the Nrf2 gene as well as the presence of a heterozygous 6/7 sequence in the downstream Nrf2-dependent phase II gene UGT1A1 potently down-regulated an individual's ability to respond to coffee with increased antioxidative gene transcription.

Here, we have demonstrated downstream effects on transcriptional regulation within the antioxidative phase II pathway dependent upon the genotype of the key transcriptional regulator Nrf2. We therefore suggest that genotype must be considered when determining criteria to evaluate treatment and outcome strategies in human antioxidant interventions and therapies. Specifically, genotype needs to be considered when targeting Nrf2-dependent gene response in order to estimate the chemopreventive potency of bioactive food/food constituents. With phase II enzymatic activity implicated in risk of several cancers, our ability to monitor and manipulate this important pathway has enormous therapeutic potential.

Methods

Coffee brew

The study coffee brew consisted of a special roasted and blended Arabica coffee, characterized by a high concentration of both, green and roasted bean constituents, especially n-chlorogenic acid (252 mg/L) and NMP (76 mg/L). The caffeine level (720 mg/L) was in the average range of conventional coffee brews. The ground coffee was delivered in vacuum bags (12.5 g each). Immediately before consumption, the coffee brew was freshly prepared using a Senseo coffee maker (Philips). Each individual consumed 250 mL of the coffee three times a day.

Subjects

The study was approved by the Griffith University Human Research Ethics Committee (EC00162). The final test population was comprised of 25 male and 26 female (total n = 51), healthy non-smoking (age 18-61, BMI 19-38) Caucasians. Originally, 57 individuals were recruited, 6 individuals did not complete the trial for various reasons (gastric symptoms or sleeplessness after coffee consumption). All participants were regular coffee drinkers (2-6 cups of coffee/day). Participants were asked to maintain their usual dietary habits for the duration of the study, except for the coffee intake, caffeinated products, dietary supplements and foods rich in polyphenols. All volunteers were informed of the objectives of the study and consent

received for their participation. Exclusion criteria included smoking and the use of medication. In addition, competitive athletes were excluded. The 8-week intervention trial was designed as follows: weeks 1-2, 1st wash-out; weeks 3-6, coffee consumption and weeks 7-8, 2nd wash-out. During the consumption period, participants consumed 750 mL of freshly brewed coffee (with/without sugar, addition of milk up to 50 mL) in three equal portions (morning, noontime, afternoon). During the two wash-out periods, the coffee brew was replaced by equal volumes of water. Nutritional reports covering a 7-day period were completed by participants in the last week of each study period. Urine/blood sampling was performed at the beginning of the study and on the last day of each study period in the morning, after a fasting period of at least 6 hours.

Genomic DNA isolation

For genomic DNA (gDNA) isolation, the QIAamp DNA Mini kit (QIAGEN) was used and gDNA purified as per the manufacturer's instructions. The final concentration of DNA was quantified using a ND-1000 spectrophotometer (Nanodrop, Delaware).

Sequencing

SNPs rs35652124, rs6706649 and rs6721961:

To obtain the 424bp amplicon of the polymorphic region of the NFE2L2 gene, 80ng of gDNA was amplified with 4μL of GoTaq® Flexi buffer (Promega), 2.5mM $MgCl_2$, 0.2mM dNTP, 0.2μM of each NFE2L2 primer (forward primer 5'–GACCACTCTCCGACCTAAAGG–3' and reverse primer 5'–CGAGATAAAGAGTTGTTTGCGAA-3') and 0.2μL GoTaq® to a total reaction volume of 20 μL. Amplification was performed on a Veriti™ 96-well Thermal Cycler (Applied Biosystems) with an initial step of 94°C x10 min, 30 cycles of 94°C x 45 sec, 55°C x 45 sec and 72°C x 45s and a final extension of 72°C x 7 min. 5μL of PCR product was electrophoresed on 2% agarose gels stained with ethidium bromide for 30 min at 80V. PCR products were purified with the ExoSAP-IT® PCR cleanup kit (Affymetrix/USB) according to the manufacturer's instructions. DNA concentration was quantified using a ND-1000 spectrophotometer (Nanodrop, Delaware) and diluted to a concentration of 20ng/μL DNA. For the sequencing reaction 1μL of DNA was added to 5μL of BigDye Terminator v3 (BDT v3.1, Applied Biosystems), 1.3μL of each NFE2L2 primer and 3.0μL of 5X BDT v3.1 Sequencing buffer. Amplification

cycles were as follows: 96°C x 1 min, 30 cycles of 96°C x 10 sec, 50°C x 5 sec and 60°C x 4 min followed by 4°C x 5 min, 10°C x 5 min and 4°C x 2min. The product was transferred to a 1.5ml tube and ethanol precipitated by adding 2 µL of iced cold 3M sodium acetate (pH 5.2) and 2 µL of 125mM EDTA (pH 8.0). The sample was vortexed, centrifuged at 10,000 g for 5 min, followed by addition of 50 µL of 100% ethanol followed by repeated vortexing and re-spinning the samples at 10,000 g for 5 min. Samples were then incubated for 15 min (RT), precipitated by centrifugation at 10,000g followed by incubation at 4°C for 20 min. The pellet was then rinsed in 70% ethanol, vortexed and centrifuged at 10,000g at 4°C for 5 min. The pellet was dried using a DNA Speed Vac® (Savant) on high drying mode for 5 min. Samples were then resuspended in 15µL dH_2O. The purified products were directly added into a Micro Amp^{TM} optical 96 well reaction plate (Applied Biosystems) to be analyzed on a 3130 genetic analyzer (Applied Biosystems).

GST1A1 genotyping

GST1A1 genotyping was carried out by RFLP analysis. For PCR amplification, gene-specific primers (forward primer 5'-TGTTGATTGTTTGCCTGAAATT-3'; reverse primer 5'-GTTAACGCTGTCACCGTCCT-3') were used to generate a 480bp PCR product spanning the polymorphism. For the amplification of GSTA1 40ng of gDNA was amplified with 4µL of GoTag® Flexi buffer (Promega), 2.5mM $MgCl_2$, 0.2mM each dNTP, 0.1µM of each GST1A1 primer and 1U Taq polymerase added. Amplification was performed on a VeritiTM 96-well Thermal Cycler (Applied Biosystems) with an initial denaturation of 94°C x 10 min, 30 cycles of 94°C x 45 s, 58°C x 45 s and 72°C x 45s, and a final extension of 72°C x 7 min. PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide for 30 min at 80 V. A no template control was included to detect possible contamination issues. This PCR product was then digested with EAR1 (New England Biolabs, Beverly, Massachusetts, USA). PCR reaction products were digested using 4U of restriction enzyme *Ear1*, 2 µL of 10X Buffer and 18 µl PCR product for 5 h at 37◦C. A no template control was included. The digested products were resolved on a 4% ultrapure agarose gel post-stained with 20µL ethidium bromide for 10 min. The GST1A1*A genotype produced a band at 480bp. A heterozygote GST1A1*A/B resulted in three bands at 480, 380 and 100bp and the GST1A1*B genotype resulted in two bands at 380 and 100bp.

UGT1A1 genotyping by size fragment analysis

First, PCR was performed using one unlabelled and one 5'-end 6-FAM labelled primer (forward primer: 5'-156-FAM/AAGTGAACTCCCTGCTACCTT-3', reverse primer 5'-CCACTGGGATCAACAGTATCT-3') resulting in a 253bp PCR product, flanking the polymorphic TA locus in the promoter region. Briefly, 80ng of gDNA was amplified with 4µL of GoTag® Flexi buffer (Promega), 2.5mM $MgCl_2$, 0.2mM each dNTP, 0.1µM of each UGT1A1 primer. 1U Taq polymerase was added for a total reaction volume of 20µL. Amplification was performed on a VeritiTM 96-well Thermal Cycler (Applied Biosystems) with an initial denaturation of 94°C x 15 min, followed by 30 cycles of 95°C x 30s, 58°C x 40s and 72°C x 40s, followed by a final extension of 72°C for 7 min. Following amplification, PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide for 30 min at 80V. A no template control was included to detect any contamination. For sequencing, 0.5µL of PCR product was administered to 0.25µL 1:10 diluted GeneScan™ 500 LIZ Size Standard (Applied Biosystems) and 9.25µL of HiDi formamide (Applied Biosystems). Samples were diluted 1:5, subjected to sequencing on the 3130 genetic analyzer (Applied Biosystems) for $(TA)_n$, and scored via Gene Mapper v4.0 software (Applied Biosystems). Control DNAs from previously sequenced individuals known to have a 6/6, 6/7 or 7/7 genotype were included in the PCR analysis. The amplified product yielded a 93-or a 95-base pair fragment, which corresponded to $(TA)_6$ and $(TA)_7$, respectively.

Gene transcription by Q-PCR

At each of the four different BC points, venous blood samples were collected in EDTA-tubes and stored at RT until the sampling period was completed. RNA was then extracted from the human PBLs, and Q-PCR performed as previously reported [15]. Total RNA was extracted from isolated PBLs following the manufactures handbook of the RNeasy®Mini Kit (QIAGEN, Hilden, Germany). Following this, 2µg RNA was reverse-transcribed using Oligo-dT primers and the Omniscript® Reverse Transcription Kit (QIAGEN, Hilden, Germany). cDNA obtained from the RT reaction (amount corresponding to 2µg of total RNA) was subjected to Q-PCR using QuantiTect SYBR® Green PCR (QIAGEN, Hilden, Germany). The primer assays used were: Hs_NFE2L2_1_SG, QT00027384. ß-Actin: Hs_ACTB_1_SG,

QT00095431 GST1A1: Hs_GST1A1_1_SG, QT00060739. GSTT1: Hs_GSTT1_2_SG, QT010751638. UGT1A1: Hs_UGT1A1_1_SG, QT00020860 (QIAGEN, Hilden, Germany). Primer concentrations and Q-PCR reaction parameters were according to manufacturer's guidelines in QuantiTect SYBR® Green PCR Handbook 11/2005 (QIAGEN, Hilden, Germany). Each sample was amplified in triplicate. A no RevT control was included for all assays.

Statistical analysis

The fold changes in expression of the target gene relative to the internal control gene (ß-actin) was analyzed using Bio-Opticon Software and the C_T data was imported into Microsoft Excel 03. Data of all assays was analyzed by the $2^{-\Delta\Delta C}{}_T$ method. Data presented are the mean ± SD of the individuals, grouped according to their respective genotypes and presented as relative transcription of wash-out (BC1 or BC2) = 1. Significances indicated are calculated by Student's *t* test ($^{\#}p < 0.05$ and ***, $^{\#\#\#}p < 0.001$).

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